(15) Conjunctivitis

Growth Factors and Collagen Distribution in Vernal Keratoconjunctivitis

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PURPOSE. To study the extracellular composition of giant papillae in vernal keratoconjunctivitis (VKC) and the expression of growth factors that may stimulate fibrosis.

METHODS. Upper conjunctival specimens were obtained by biopsy in 9 patients affected by active tarsal VKC (14 eyes) and 10 normal control subjects. Immunohistochemistry was performed on tissue sections using monoclonal antibodies (mAbs) for collagens I, III, and VII; tumor necrosis factor (TNF)-α; transforming growth factor (TGF)-β1; basic fibroblast growth factor (bFGF); and platelet-derived growth factor (PDGF). The mAbs anti-tryptase, anti-CD4, anti-CD68, and anti-EG2 were used as markers for mast cells, T-helper lymphocytes, macrophages, and eosinophils, respectively. Immunofluorescent double-staining for growth factors and cell markers was performed in VKC tissues.

RESULTS. Immunostaining was highly positive for collagens I, III, and VII in the subepithelium of VKC conjunctiva. Image analysis showed a significant increase of staining per tissue area for both collagens I and VII and increased basal membrane length. The number of cells positive for TNF-α, TGF-β, bFGF, or PDGF was significantly higher in VKC tissue than in control samples. Double staining showed that eosinophils and macrophages were the main sources of PDGF and that FGF was expressed by 46% of mast cells. Significant PDGF and FGF staining was observed in the conjunctival epithelium and vascular endothelium of all VKC tissues.

CONCLUSIONS. In giant papillae of VKC, the extracellular matrix is characterized by overproduction of collagens. Expression of growth factors in the conjunctiva by resident cells (mast cells, epithelial cells, endothelial cells) and inflammatory cells (macrophages, eosinophils) may contribute to papillae formation and fibrosis evolution in chronic ocular allergic diseases. (*Invest Ophthalmol Vis Sci.* 2000;41:4175-4181)

Vernal keratoconjunctivitis (VKC) is a recurrent or chronic ocular inflammatory allergic disease that affects children and young adults, particularly those living in warm climates. There are two clinical forms, tarsal and limbal, the former of which is characterized by the formation of giant papillae, usually on the upper tarsal plate and, only rarely, on the inferior tarsal conjunctiva. Only in severe limbal forms of the disease do fibrous proliferations develop at the limbus. Similar scar tissue formation is a common consequence of other chronic allergic conditions, such as asthma and nasal polyposis.[1,2] Giant papillae in the upper tarsal conjunctiva are the result of architectural remodeling of the conjunctiva and, presumably, of increased deposition of extracellular matrix components. Chronic allergic inflammation in VKC is characterized mostly by an intense eosinophil infiltration; increased number of mast cells, basophils, neutrophils, and macrophages; and the presence of predominately T-helper type 2 (Th2) lymphocytes.[3-6]

Previous studies of VKC have demonstrated an increased presence of collagens I and III in giant papillae,[7] and procollagens and cytokines in tears, the latter of which may stimulate fibroblast proliferation and collagen production.[8] Fibroblasts respond to various microenvironmental factors including soluble cytokines and growth factors, as well as cell–matrix and/or cell–cell interactions that intervene significantly in the control of synthesis and degradation of the extracellular matrix.[9] In allergic disorders, tissue remodeling may be driven by cytokines and growth factors released by activated resident cells such as mast cells, fibroblasts, vascular endothelial cells, and epithelial cells and by activated inflammatory cells such as eosinophils and mononuclear cells. In particular, mast cells and eosinophils, which are prominent features in the histopathology of ocular allergic diseases, may modulate the extracellular matrix metabolism through the release of their mediators. In vitro studies on fibroblasts derived from VKC tissues demonstrated their hyperproliferative capacity in response to histamine,[10] epidermal growth factor (EGF), transforming growth factor (TGF)-β1, and basic fibroblast growth factor (bFGF; Leonardi A, unpublished data, 2000).

TGF-β1, bFGF, and platelet-derived growth factor (PDGF) are multifunctional polypeptides that affect the growth and differentiation of a broad spectrum of cell types. The biologic

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activity of these molecules may be relevant to the structural changes in VKC driven by the proliferation of fibroblasts, epithelial cells, and vascular endothelial cells and the deposition of extracellular matrix proteins. These three growth factors have been shown to play a relevant role in the remodeling process of nonocular allergic diseases, such as asthma and nasal polyposis.[9,11]

The purpose of the present study was to characterize the composition and distribution of collagens in giant papillae of VKC and to evaluate by immunohistochemistry the expression and cell source of cytokines and growth factors such as tumor necrosis factor (TNF)-α, TGF-β1, bFGF, and PDGF. Mast cell-derived TNF-α and TGF-β1 have been implicated in the promotion of fibroblast proliferation and collagen expression in a coculture system of murine mast cells and fibroblasts.[12,13] These molecules may contribute to fibroblast stimulation and thus to giant papillae formation in this disease of chronic allergic inflammation.

Materials and Methods

Subjects

Under local anesthesia, conjunctival specimens were obtained by biopsy from the upper tarsal conjunctiva of 14 eyes of 9 patients with active VKC (7 males; mean age 13.5 years, range 7–21 years) and from 10 eyes of 10 normal subjects (mean age 45.2 years, range 14–60 years): 7 at the time of surgery for ptosis ($n = 2$), chalazion ($n = 3$), or cataract ($n = 2$) and 3 from the eye bank ($n = 3$). The research followed the tenets of the Declaration of Helsinki. After explanation of the nature and possible consequences of the study, informed consent was obtained from all subjects or parents in the case of minors. The control group was purposefully chosen with a wide range of ages. No subject in the control group had a history of contact lens wear or any inflammatory signs and symptoms. Diagnosis of tarsal VKC was based on clinical history and signs and symptoms. All patients with VKC were positive to skin tests and/or to specific IgE in serum for common environmental allergens. In all patients, therapy had been suspended for at least 5 days before biopsy. Bioptic tissues were snap frozen with optimal cutting temperature compound (OCT; Miles, Elkhart, IN) in liquid nitrogen and maintained at −70°C.

Immunostaining

Serial 7-μm-thick cryosections were cut by ultramicrotome, mounted on gelatin-covered slides, fixed in acetone for 10 minutes, and processed for immunohistochemistry. The following anti-human monoclonal or polyclonal antibodies were used: for extracellular matrix components: anti-collagen I 1:2000, anti-collagen III 1:1000, anti-collagen VII 1:1000, and anti-fibronectin 1:1000 (all from Sigma, St. Louis, MO); for cytokines and growth factors: anti-TNF-α 1:20 (Serotec, Oxford, UK), anti-TGF-β1 1:500 (Serotec), anti-PDGF-BB 1:50 (Genzyme, Cambridge, MA), and anti-bFGF2 1:50 (Chemicon, Temecula, CA); inflammatory cell markers: anti-tryptase 1:50 (AA-1, Dako, Glostrup, Denmark) specific for mast cells, anti-eosinophil cationic protein (anti-ECP) 1:50 (clone EG2, Pharmacia & Upjohn, Uppsala, Sweden), anti-CD4 1:50 (Dako) specific for T-helper lymphocytes, and anti-CD68 1:100 (Dako) specific for macrophages.

Briefly, for all mAbs, unspecific binding was blocked by the addition of serum derived from the same animal species as the secondary antibodies. The slides were washed twice in Tris-buffered saline (TBS), and the appropriately diluted anti-human antibodies were placed on each tissue section and incubated in a moist chamber for 60 minutes. After primary incubation, the slides were washed in TBS, incubated for 30 minutes with the secondary antibodies, and treated with alkaline phosphatase complex (APAAP; Dako). The reaction was developed with fast red solution and counterstained with Mayer's hematoxylin.

The avidin-biotin complex technique (Vectastain-ABC kit; Vector, Burlingame, CA) was used to reveal the immunoreaction of sections treated with the rabbit polyclonal antibodies for PDGF-BB and bFGF. Sections were developed for 20 minutes in peroxide substrate solution containing 3,3′-diaminobenzidine and hydrogen peroxide and were counterstained with Mayer's hematoxylin. Negative control specimens were prepared using sections incubated without the primary antibody. Positive control specimens were prepared using tissue sections of placenta for extracellular matrix components and growth factors and sections of lymphoid tissue for cytokines and inflammatory cell markers.

The positive red or brown reaction in the epithelium and in the extracellular space of the stroma was analyzed in five representative fields (×250) from VKC and normal specimens. It was classified as very intense, intense, slight, or absent. Intracellular staining for TGF-β, TNF-α, bFGF, and PDGF in the epithelium and in the substantia propria was quantified by cell counts in 10 high-power fields (×400). A 10 × 10-mm grid was used for each assessment under a microscope (Carl Zeiss, Oberkochen, Germany), and all quantifications were performed in a masked fashion. The cell number is expressed as cells per square millimeter of tissue.

To verify the expression of PDGF and FGF from eosinophils, macrophages or mast cells, a two-color indirect immunofluorescence technique was performed in three patients with VKC. The binding of rabbit polyclonal antibodies against PDGF and FGF was shown using goat anti-rabbit IgG-fluorescein isothiocyanate (Vector) and that of mouse mAb against EG2, CD68, or tryptase by incubation with Texas red anti-mouse IgG (Vector). Briefly, the slides were incubated with the primary antibody in phosphate-buffered saline (PBS) for 1 hour and then with the secondary labeled anti-IgG antibody for 30 minutes. Negative control slides were incubated with PBS or with nonspecific IgG mAb.

Table 1. Immunohistochemistry of Collagens and Fibronectin in VKC and Controls

	Subepithelium		Stroma	
	VKC	CT	VKC	CT
Collagen I	+++	+	+++	+
Collagen III	++	+	+	−
Collagen VII	+++	+	−	−
Fibronectin	+	+/−	+	−

+, slight; ++, intense; +++, very intense; −, absent; CT, control.

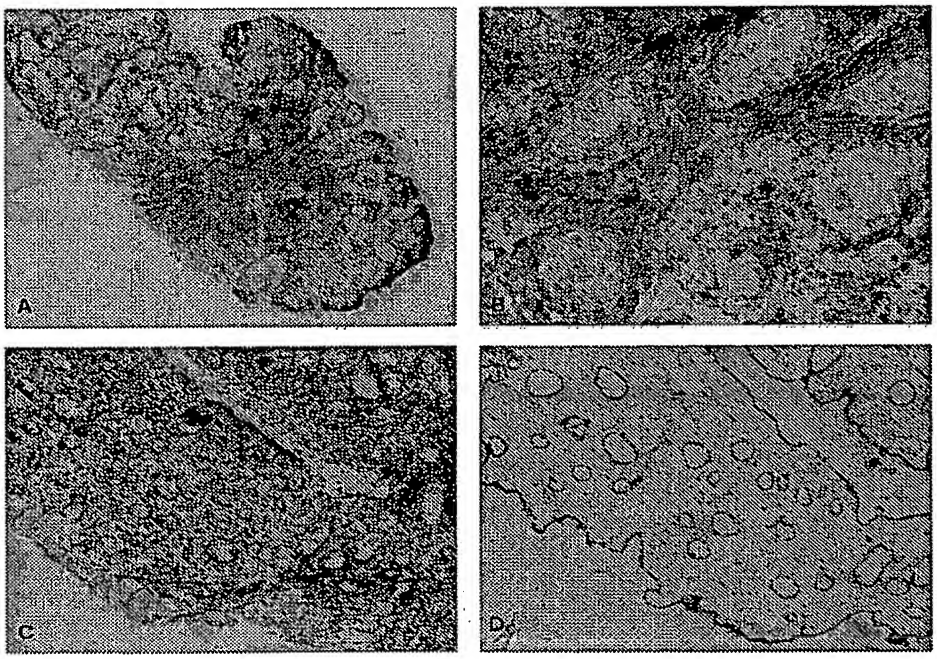

FIGURE 1. Immunostaining for collagens in VKC conjunctival tissues: (A) low magnification of a giant papillae section showing collagen I distribution. (B) Collagen I bundles around the epithelial ingrowths (×400). Patterns of immunostaining for collagen type I (C) and VII (D) in the same section of giant papillae (×200).

Morphometric Analysis

Immunoreactive areas for collagen I were evaluated in the whole tissue with a computerized image analyzer (AT-IBAS; Kontron, Eching, Germany) connected to both a black-and-white video camera (TYK9B; Bosch, Germany) and a light microscope (UMSP80; Carl Zeiss), using ×25 as the primary magnification. Five fields were analyzed for each tissue section. Digitized images (512 × 512 pixels, 256 gray levels) were processed with shading corrected by increasing contrast. Images were then processed with dynamic thresholding to better discriminate the immunoreactive area. The ratio of positive to whole tissue area was used for statistical analysis. A similar procedure was used to analyze the distribution of collagen VII. In this case, the length of the immunoreactive area, and thus the ratio of membrane to tissue area, was also evaluated.

Statistics

Differences between cell counts and morphometric numerical results in VKC versus control tissues were analyzed using the Mann–Whitney test and Student's *t*-test. Results are illustrated as the mean ± SD. Statistical significance was set at $P \leq 0.05$.

RESULTS

Light microscopy evaluation of VKC tissues was characterized by numerous epithelial ingrowths in an irregular hyperplastic mucosa infiltrated by mixed inflammatory cells. Epithelial infoldings, pseudotubuli, and crypts had a high density of goblet cells. Eosinophils, neutrophils, and mononuclear cells infiltrated the well-preserved hyperplastic epithelium. Areas of epithelial cell loss were also observed. The hyperplastic extra-

TABLE 2. Morphometric Analysis of Collagens I and VII in VKC and Control Tissues

	Collagen I		
	Positive Area	Tissue Area	Positive Area/Tissue Area Ratio
VKC	7,564.5 ± 599	26,032.05	0.228 ± 0.023
CT	2,395.7 ± 175	26,032.05	0.092 ± 0.007
P	0.0016	NS	0.0016

	Collagen VII				
	Positive Area	Length of Membrane	Tissue Area	Positive Area/Tissue Area Ratio	Membrane/Tissue Area Ratio
VKC	1,517 ± 413	59.6 ± 10.2	19,725 ± 845	0.076 ± 0.02	0.03 ± 0.001
CT	571.8 ± 195	51.5 ± 0.4	20,217 ± 268	0.029 ± 0.01	0.03 ± 0.0003
P	0.0016	0.0024	NS	0.001	0.001

Data are positive red reaction per square micron of tissue (positive area), and micron (length). CT, control tissues; NS, not significant.

TABLE 3. Number of Positive Cells in VKC and Control Conjunctival Stroma

	Tryptase	CD4	CD68	EG2	TNF-α	TGF-β1	bFGF	PDGF-B
VKC	79.9 ± 22	270.5 ± 70	140.1 ± 25	190.9 ± 51	49.2 ± 10.8	49 ± 19	223.5 ± 37	170.6 ± 32
CT	35.6 ± 24	33.5 ± 7.9	58 ± 13	0.2 ± 0.3	5 ± 1.4	0	58.7 ± 24	0
P	0.003	0.001	0.001	0.0017	0.013	0.001	0.0006	0.009

Data are expressed as mean number of cells per square millimeter.

cellular matrix appeared organized in gross fibers beneath the epithelium and was irregularly distributed in the deep stroma. A massive inflammatory cell infiltrate was noted around the blood vessels and in the subepithelium, occasionally forming a pseudofollicle without a germinative center.

Immunohistochemistry demonstrated collagen type I to be the main constituent of the extracellular matrix in both VKC and control specimens (Table 1). Immunostaining was highly positive in the subepithelium of VKC specimens. In this area, collagen fibers laid parallel to the epithelial surface with perpendicular fibers radiating more deeply into the stroma (Fig. 1). When epithelial ingrowths were cut perpendicularly, an annular collagenous architecture appeared to support the epithelial structure. At the root of the papillae, a central fibrovascular core was evident with a ramified fibrotic formation growing out of it (Fig. 1).

Slight immunohistochemical staining for collagen III and fibronectin was observed in normal conjunctiva and in increased amounts in VKC (Table 1). Both were particularly evident in the subepithelium of VKC tissues and along the collagen bundles.

Collagen type VII was characteristically found in the basal membrane of both normal subjects and patients with VKC. In normal subjects, it was disposed linearly and parallel to the surface, whereas in patients with VKC, it was localized more in the epithelial ingrowths in the deep stroma (Fig. 1).

Image analysis showed a significant increase of the ratio of staining to tissue area for both collagens I and VII ($P < 0.001$) and increased basal membrane length ($P < 0.01$) (Table 2). Because of weak positive staining, image analyses of collagen III and fibronectin were not performed.

Immunohistochemistry for growth factors showed a strong diffuse epithelial staining for TGF-β, PDGF, and bFGF in VKC samples that was absent or weak in control samples. This staining was stronger in the basal epithelial layer, both in the cytoplasm and on the cell membrane. TNF-α was negative in the epithelium of both VKC and control specimens. Tryptase-positive cells (mast cells) in the epithelium were found only in VKC tissue. Their number was significantly increased compared with control specimens (184 ± 66 cells/mm^2 versus 0 ± 0 cell/mm^2; $P = 0.001$). No eosinophils (EG2$^+$) were found in the epithelium of control specimens, but several helper lymphocytes (CD4$^+$) and macrophages/dendritic cells (CD68$^+$) were observed.

In the conjunctival stroma, the numbers of mast cells, EG2$^+$, CD4$^+$, and CD68$^+$ cells were significantly increased in VKC compared with control specimens (Table 3). The number of cells positive for TNF-α, TGF-β1, PDGF, or bFGF was also significantly higher in VKC tissues compared with control specimens (Table 3, Figs. 2 and 3). Blood vessel endothelial cells were positive for TGF-β, bFGF, and PDGF in VKC samples and weakly positive in control specimens.

Extracellular conjunctival stroma was positive for TGF-β, bFGF, and PDGF only in VKC samples. The staining was stronger along the collagen fibers, especially for PDGF. TGF-β was particularly evident around blood vessels and epithelial ingrowths. No extracellular staining for TNF-α was found in either VKC or normal specimens.

Double immunofluorescence techniques showed that 71% ± 13% of CD68$^+$ cells were also positive for PDGF (Fig. 4). Conversely, 50% ± 17% of PDGF-positive cells were also positive for CD68. Double immunofluorescence for PDGF and EG2 showed that 38% ± 8% of activated eosinophils expressed PDGF, whereas only 25% ± 14% of PDGF$^+$ cells were also EG2$^+$. Double immunofluorescence for FGF and CD68 showed that 37% ± 12% of FGF-positive cells were CD68$^+$ and that 40% ± 10% of CD68$^+$ cells were also positive for FGF. Double

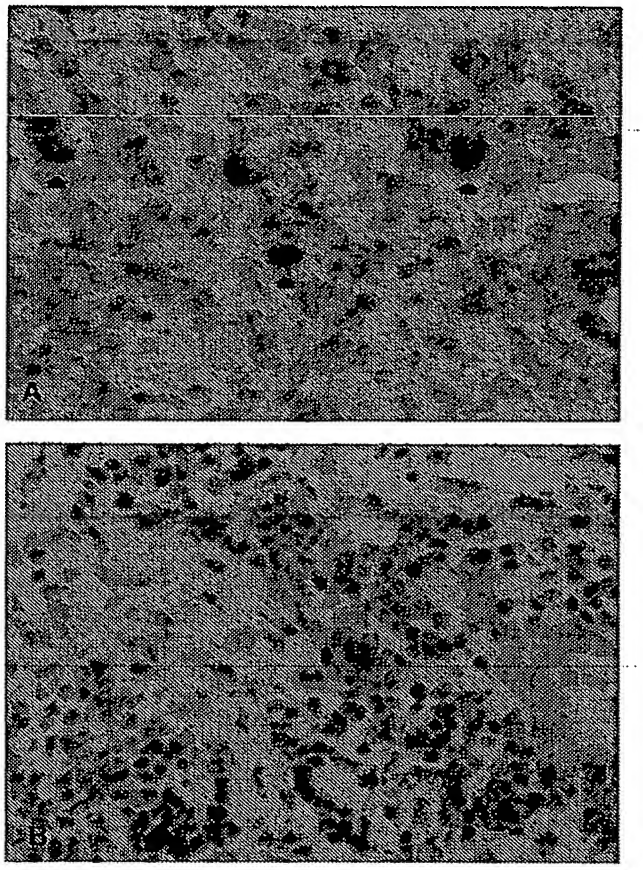

FIGURE 2. Intracellular positive immunostaining (*arrows*) for TGF-β1 (A) and TNF-α (B) in VKC tissues. Magnification, ×400.

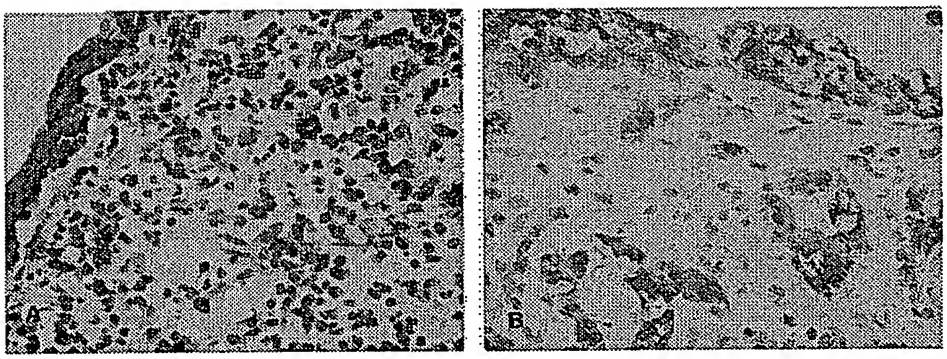

FIGURE 3. Immunostaining for PDGF (A) and FGF (B) in the epithelium and in a subpopulation of cells in the stroma of VKC tissues. Magnification, ×400.

staining for FGF and tryptase showed that 17% ± 9% of FGF-positive cells were mast cells and that 46% ± 15% of mast cells were positive for FGF. Epithelial and vascular endothelial cells were positive only for PDGF or FGF, indicating that the first stain for macrophages, eosinophils, or mast cells did not cross-react with the second stain.

DISCUSSION

Pathologic fibroblast proliferation or tissue fibrosis develops in certain chronic allergic diseases and in a wide spectrum of inflammatory disorders in which mast cells and eosinophils are a prominent feature. Giant papillae in VKC are a morphologic expression of fibroblast activation and tissue remodeling in a chronic allergic disorder in which mast cells, eosinophils, and Th2 lymphocytes are known to play a pivotal role. In this study, the overproduction of growth factors such as TGF-β1, PDGF, and bFGF was shown for the first time to be associated with an increased deposition of collagen in the giant papillae of VKC.

Collagen type I seemed to be the predominant connective tissue element in VKC conjunctival tissues. Immunolocalization of collagens I and III and fibronectin was particularly evident in the subepithelial region, from which thick collagen I fibers radiated in an irregular fashion deep into the stroma. In previous studies, we reported an increased number of fibroblasts in VKC giant papillae, an increased quantity of collagens I and III determined by immunoelectrophoresis,[7] and increased levels of procollagens I and III in tears and in tissue cultures from patients with VKC with giant papillae.[8] In the present study, the distribution of collagens I and III was better defined. Morphometric analyses, which allowed for the numerical quantification of collagen, demonstrated that collagens I and VII were significantly increased per tissue area.

In contrast to collagen IV, the main constituent of basal membrane, collagen VII is not visualized in blood vessels. Collagen VII is a primary structural element of anchoring fibrils

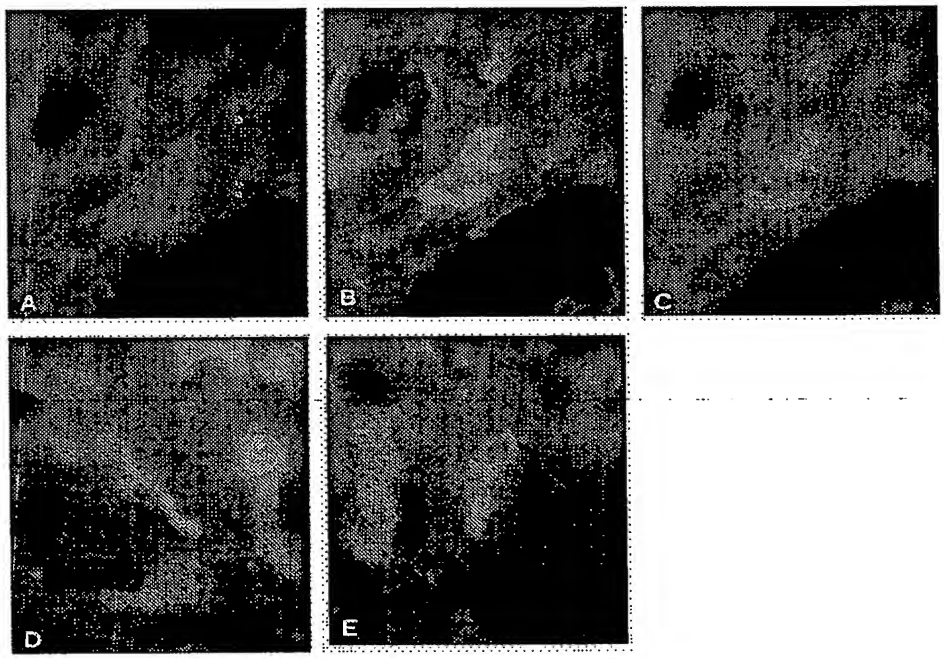

FIGURE 4. Double immunofluorescence in VKC tissues. Positive staining for EG2 (A; *red*) and PDGF (B; *green*) demonstrated that eosinophils expressed PDGF (C; *orange*). Positive staining for both tryptase (*red*) and FGF (*green*) demonstrated that mast cells expressed FGF (D; *orange*). Positive staining for CD68 and PDGF demonstrated that macrophages expressed PDGF (E; *orange*).

of the basal membrane, and its presence is considered an index for increased epithelial growth and conjunctival surface area. Increased immunostaining for collagen V has also been described in the conjunctiva of patients with VKC and of those with active trachoma,[14] suggesting that other collagens may also be involved in fibrotic conjunctiva. Unquestionably, these collagens are increased by activated fibroblasts. However, additional mechanisms may add to this accumulation of extracellular matrix protein. Inhibition or reduced activity of collagenase enzymes and enhanced activity of inhibitors still unexplored in VKC are possible contributing factors.

In the present study, the expression of fibrogenic growth factors such as PDGF, bFGF, TGF-β1, and the cytokine TNF-α was increased compared with expression in normal conjunctival mucosa. These growth factors have previously been shown to induce fibroblast proliferation and extracellular matrix production in pulmonary fibrosis.[11] Furthermore, mast cell–derived TGF-β1 and TNF-α have been shown to stimulate fibroblast proliferation and collagen type I mRNA expression in a coculture system of mast cells and 3T3 fibroblasts.[12,13] Eosinophils, the most common inflammatory cells in VKC, can also produce and release TGF-β 1,[15] TNF-α,[16] and PDGF,[17] all of which may mediate fibroblast activation. In fact, it has been reported that eosinophils increase lung and dermal fibroblast proliferation and collagen production in vitro.[18,19] Conversely, fibroblast-derived cytokines and growth factors, such as IL-3 and granulocyte-macrophage colony-stimulating factor (GM-CSF), modulate and stimulate growth and survival of eosinophils and mast cells.[9,20,21] Thus, the increased expression of these growth factors in VKC tissues may explain both the massive presence and the persistence of eosinophils and mast cells and the proliferative changes in chronically inflamed tissues.

The numerous mast cells in VKC are one possible source of TGF-β1 and TNF-α. The morphology, distribution, and number of cells positive for these factors were not only similar, but also paralleled the number of mast cells in the tissue. The increased number of cells positive for PDGF and bFGF demonstrates that multiple cell types may produce these growth factors in VKC. Seventy percent of macrophages and almost 40% of eosinophils were shown to produce PDGF by double immunofluorescence. Furthermore, 40% of macrophages and 46% of mast cells in VKC conjunctiva were shown to express the growth factor bFGF. Mast cells have also been shown to be a source of bFGF in chronic nonocular inflammation.[22]

Positive staining of the stroma for PDGF, bFGF, and TGF-β1 is in accordance with previous reports.[22] It is possible that binding of growth factors to components of the extracellular matrix such as glycosaminoglycans may prolong the local activity of these molecules at the site of fibrosis. It is also notable that T-helper lymphocytes involved in VKC pathogenesis express and produce Th2 type cytokines (interleukin [IL]-4, IL-5, and IL-13), whereas interferon (IFN)-γ expression in VKC is low or absent.[3–6] This latter cytokine was shown to inhibit fibroblast proliferation and collagen production from Tenon's capsule fibroblasts in culture,[23] whereas IL-4 was shown in other studies to induce proliferation of lung fibroblasts.[24,25]

In the present study, the number of $CD4^+$ cells in VKC and normal conjunctiva was similar to that previously reported.[6] Although no double staining was performed for T-helper cells, the distribution of growth factor–positive cells in the tissues appeared to be distinct from that of $CD4^+$ cells. Nevertheless, a possible influence of Th2 cells on tissue remodeling in VKC may be mediated directly by Th2 cytokines or indirectly through T-cell–driven eosinophil and mast cell activation.

The conjunctival epithelium is both directly and indirectly involved in the development of conjunctival inflammation. When stimulated, epithelial cells can express and produce several cytokines, chemokines, and adhesion molecules that can affect chronic inflammation.[26–28] In the present study, the epithelium in VKC showed intense immunostaining for PDGF, bFGF, and TGF-β1, suggesting that these growth factors are produced by the epithelial cells or that these proteins may have been sequestered within the epithelium from tears or other sources. Intracytoplasmic immunofluorescent staining of both PDGF and bFGF was seen in the basal and superficial layers of epithelium and epithelial ingrowths, suggesting epithelial production of these factors. Because both PDGF and TGF-β1 have been shown to mediate mast cell chemotaxis,[29] it is possible that in VKC these factors may be involved in the great migration of mast cells to the epithelium of patients with VKC. Conversely, the increased number of mast cells in the epithelium may also be a stimulus for epithelial overgrowth in VKC, because mast cell–derived mediators have been shown to be mitogenic for epithelial cells in vitro.[30] It has been suggested that conjunctival epithelial cells and fibroblasts communicate through active cytokine and growth factor production, thereby regulating cellular processes such as epithelial differentiation, wound healing, and angiogenesis.[31,32] In a three-dimensional in vitro model, damaged bronchial epithelial cells have been shown to produce growth factors such as PDGF, bFGF, and TGF-β1, thus enhancing fibroblast proliferation activity.[33] Because epitheliotoxic insults, especially from activated eosinophils, are clinically evident in VKC, the production of these growth factors from conjunctival epithelial cells may initially have an autocrine or paracrine repair function, with their eventual overexpression then causing tissue remodeling. However, other growth factors and hormones may also be involved in the pathogenesis of tissue remodeling in VKC.

In conclusion, overproduction of growth factors by epithelial cells, inflammatory cells and structural cells may be responsible for the epithelial overgrowths and ingrowths and the stromal extracellular matrix expansion that characterize the giant papillae of VKC. Further in vitro studies on the interaction between activated inflammatory cells and structural cells may elucidate the pathophysiology of tissue remodeling that characterizes chronic allergic diseases.

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